Card15 gene polymorphisms in patients with spondyloarthropathies identify a specific phenotype previously related to crohn's disease

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CARD15 GENE POLYMORPHISMS IN PATIENTS WITH SPONDYLOARTHROPATHIES IDENTIFY A SPECIFIC PHENOTYPE PREVIOUSLY RELATED TO CROHN'S DISEASE

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ABSTRACT

Objective Association between spondyloarthropathy (SpA) and Crohn's disease (CD) is a well-known phenomenon. A risk for evolution to CD was already demonstrated in the subgroup of SpA patients with associated chronic gut inflammation. We investigated whether the reported polymorphisms in the *CARD15* gene, a susceptibility gene for CD, are associated with the presence of preclinical intestinal inflammation observed in SpA.
Methods We included 104 SpA patients who underwent an ileocolonoscopy with biopsies between 1983 and 2004. Using RFLP-PCR, we assessed the prevalence of three single nucleotide polymorphisms in the *CARD15* gene (R702W, G908R and 1007fs) and compared them to an ethnically matched CD population and a control population.
Results The carrier frequency of R702W, G908R or 1007fs variants in the SpA populations (20%) was similar as in the control population (17%), but increased to 38% in the subgroup of SpA patients with chronic gut inflammation. This was significantly higher than in the other SpA subgroups (P=0.001) and the control group (P=0.006) but not significantly different from the prevalence in CD (49%). This indicates that *CARD15* polymorphisms are associated with a higher risk for development of chronic gut inflammation.
Conclusion CARD15 gene polymorphisms clearly identify a subgroup of patients with SpA associated with chronic intestinal inflammation.

Key words: Spondyloarthropathy, Crohn's disease, *CARD15,* intestinal inflammation

INTRODUCTION

The spondyloarthropathies are a group of interrelated inflammatory diseases characterised by a pauciarticular, peripheral, asymmetrical arthritis and/or axial involvement with ankylosing spondylitis (AS) as prototype [1]. Reported prevalences of spondyloarthropathies vary between 0.2 and 1.9% [2, 3]. Although association with HLA-B27 is strong, recent genetic studies suggest a polygenic model of susceptibility [4-7].

In up to 60% of spondyloarthropathy (SpA) patients, articular involvement is associated with subclinical histological evidence of chronic or acute gut inflammation in ileum or colon [8-10]. We described a long-term evolution to overt Crohn's disease (CD) in 13% of patients with initial chronic gut inflammation [11, 12]. The presence of chronic intestinal inflammation did not relate to HLA-B27, but a weak association was found with HLA-B62 [11].

The observed immunologic similarities in SpA with gut inflammation and CD support the concept that this subgroup of SpA patients can be considered as a model for early immune alterations related to CD. An enrichment of gut mucosal T cell lines with αEβ7 integrin and an increased expression of its ligand, E-cadherin, was found in intestine of CD as well as SpA patients [13-15]. Recirculation of T cells primed in the gut to synovial tissue is one potential mechanism by which gut and synovial inflammation could be linked. This hypothesis is supported by an altered expression of β7 integrins, which are highly expressed within the gut, on synovial T cells from SpA patients compared to rheumatoid arthritis [16]. Another potential mechanism includes trafficking of antigen presenting cells between gut and joint. Consistent with this was the augmented infiltration of gut mucosa and synovium with CD163 positive macrophages (producing IL-1 and TNF-α) in both CD and SpA patients [17, 18]. Finally, a comparable beneficial clinical effect of infliximab, a monoclonal antibody to TNF-α suggests a key role of this cytokine in both diseases [19, 20].

In 2001, a correlation was reported between polymorphisms in the *CARD15* gene and an increased susceptibility for CD [21-23]. Three independent single nucleotide polymorphisms (SNPs) in *CARD15* are associated with CD in about 30 to 46 percent of patients (1 frame-shift mutation, 1007fs (SNP13), and 2 missense mutations, R702W (SNP8) and G908R (SNP12)) [22, 24]. These variants increase the risk for CD by a factor of 3 for heterozygous and by a factor of 38 or 44 for respectively homozygous or compound heterozygous individuals [22]. Lower prevalences have been described in CD patients in Scotland, Ireland and Northern Europe, whereas no association could be found in Japan [25-28].

CARD15 encodes for an intracellular protein, which is expressed in monocytes, granulocytes, dendritic, epithelial and Paneth cells, and has binding affinity for bacterial cell wall components like muramyldipeptides [29]. The *CARD15* protein is involved in NF-κB activation and in apoptosis by two N-terminal Caspase Recruitment Domains (hence the term CARD), although its precise pathogenetic role in CD remains to be determined [29-31].

CARD15 gene polymorphisms have also been linked with another related syndrome, Blau's syndrome, characterized by granulomatous inflammation of uvea, skin and joints [32].

Several studies have been performed to investigate the role of *CARD15* polymorphisms in SpA. These studies did not demonstrate an association with SpA or AS in particular [33-37]. Yet, an increased prevalence of *CARD15* polymorphisms was found in psoriatic arthritis but not in psoriatic skin disease [38-40]. A recent Italian study however could not confirm this association [41]. Nevertheless, this finding could emphasize the importance of investigating the possible role of these genetic variants in specific, clinical subpopulations of patients. In CD as well, *CARD15* polymorphisms seem to be related with certain clinical phenotypes [42-46].

In view of the apparent correlation between gut inflammation in SpA and clinical evolution to CD, we investigated whether the presence of polymorphisms in this susceptibility gene for CD would be associated with gut inflammation in SpA patients.

MATERIALS AND METHODS

Study population.

This study included 104 Caucasian SpA patients (according to the ESSG criteria [47]), who underwent an ileocolonoscopy with concomitant ileal and colonic biopsies between 1983 and 2004. This population consisted of 74 male and 30 female patients with a mean age of 46 years (range: 21-77 y). SpA patients were systematically referred by the rheumatologist for an ileocolonoscopy with biopsies, independent of the presence of GI symptoms.

Patients with the diagnosis of clinical Crohn's disease or psoriasis prior to the diagnosis of SpA, were excluded from the study.

A subgroup of 54 patients, all having a longterm follow-up since their diagnosis of SpA (ranging from 17 to 49 years), was recently clinically reassessed. New follow-up colonoscopies were not performed.

The total SpA population consisted of 75 patients with ankylosing spondylitis (AS) according to the modified New York criteria [48] and 29 patients with an undifferentiated form of SpA (uSpA). Eighteen AS patients only had axial involvement, whereas 57 AS patients also had peripheral disease (defined as the history or presence of peripheral arthritis and/or enthesitis). Twenty-five uSpA patients had peripheral disease and 4 uSpA patients only had axial involvement. These 4 patients had inflammatory low back pain and fulfilled the ESSG criteria, however not the modified New York criteria for AS.

HLA-B27 status was known in a total of 81 patients. In 53 patients both HLA-B27 and HLA-B62 status were known.

A population of 156 consecutive patients with proven CD on clinical, endoscopical and histological grounds was included as well. This cohort included 57 male and 99 female patients with a mean age of 38 years (range: 18-80 y).

Prevalences were also compared to those observed in a control population including 140 individuals.

The study was approved by the local ethics committee. All patients signed an informed consent.

Histological classification.

A classification of histologic lesions was used as reported in previous studies [8, 11, 12, 49, 50]. Three subgroups were distinguished: patients with normal gut histology, acute and chronic inflammation [51]. In acute inflammatory lesions normal architecture was well preserved. A mucosal and epithelial infiltration by neutrophils and eosinophils was found, without a significant increase in lymphocytes. Small superficial ulcers covered with fibrin and neutrophils overlying hyperplastic lymphoid follicles were occasionally observed. The lamina propria was oedematous and hemorrhagic and contained mainly polymorphonuclear cells. The pattern of inflammation was similar to that seen in acute self-limiting bacterial enterocolitis.

The principal features of chronic inflammatory lesions were mucosal architectural alterations with crypt distortion and atrophy in the colon and villous blunting and fusion in ileal mucosa. Both in ileum and colon there was an increased mixed cellularity and formation of basal lymphoid aggregates in the lamina propria.

Whenever one of several biopsies featured chronic lesions, regardless of acute or active inflammation in other fragments, a diagnosis of chronic inflammation was made.

Although NSAID may induce intestinal disorders, we and others excluded these drugs as aetiology of reported chronic inflammation [8, 10, 52].

CARD15 genotyping (R702W, G908R and 1007fs), HLA-B27 and HLA-B62 typing.
Genomic DNA was extracted from whole blood using Qiagen blood and cell culture DNA kit (Westburg BV, Leusden, The Netherlands) and genotyped all patients for R702W, G908R and 1007fs using RFLP-PCR, followed by separation of the DNA fragments on a 2.5% agarose gel. The missense mutation R702W (GenBank accession number G67950) abolishes the restriction site for *MspI*, resulting in an intact 130-bp band for mutant alleles compared to two bands of 54- and 76-bp for wild type alleles (forward primer: 5'-CAGCCCTGATGACATTTCTCTT-3', reverse primer: 5'-AGCCGCTCCTCCTGCATCTCGTA-3'). The missense mutation G908R (GenBank accession number G67951) creates a restriction site for *HinP1I*. The frameshift mutation 1007fs (GenBank accession number G67955) creates a restriction site for *NlaIV*. The presence of a mutant allele results in two bands of 219 and 41 bp, while the wild type allele produces a single 260-bp product (forward primer: 5'-CTGAGCCTTTGTTGATGAGC-3', reverse primer: 5'-TCTTCCAACCACATCCCCATT-3').
In the patients with a known HLA-B27 and HLA-B62 status, typing of these markers had been performed using the microlymphocytotoxity test according to Terasaki and McClelland [53].

Statistical Analysis.
Statistical significance was determined by the Chi-square test and Odds Ratio using SPSS (SPSS inc., Chicago, Illinois). Multivariate analysis (logistic regression) was performed to investigate whether an association, found through univariate analysis, was independent from other genetic markers. *P* values less than 0.05 were considered significant.

RESULTS

We subdivided our cohort in three groups according to the gut histology. Forty patients (38%) had a normal histology, 24 patients (23%) had acute gut inflammation and 40 (38%) showed chronic gut inflammation (Table 1).

Table 1. Prevalence of *CARD15* variants in the populations, according to subtypes defined at baseline.

	n	carriers of *CARD15* variant(s)
Classification		
Control population	140	24 (17%)
Crohn population	156	77 (49%)*
Spondyloarthropathy (SpA) population	104	21 (20%)§
Ankylosing Spondylitis (AS)	75	16 (21%)
Undifferentiated SpA (uSpA)	29	5 (17%)
Gut histology in SpA population		
chronic inflammation	40 (38%)	15 (38%)‡
acute inflammation	24 (23%)	0 (0%)
normal histology	40 (38%)	6 (15%)

* Chi-square: *P<0.001* (carrier frequency in CD vs control population)
§ Chi-square: *P*=0.5 (carrier frequency in general SpA vs control population)
‡ Chi-square: *P*=0.001(chronic inflammation in patients with *CARD15* variant vs chronic inflammation in those without *CARD15* polymorphism)

Univariate analysis

Prevalence of CARD15 polymorphisms in the SpA, CD and control populations.

The prevalences of *CARD15* polymorphisms in the total SpA (20%), specific AS (21%) and uSpA (17%) population did not differ significantly (Table 1). All except one (homozygous for the 1007fs allelic variant) were heterozygous for at least one mutation. The prevalence of R702W, G908R and 1007fs allelic variants in this SpA population was 12%, 4% and 5% respectively (Table 2). No compound heterozygousity was found. All carriers of *CARD15* polymorphisms in the SpA group had (a history of) peripheral disease (Table 3). There were no significant differences concerning the disease duration and the duration of the follow-up period between the SpA patients carrying *CARD15* poymorphisms and the group of patients without these polymorphisms (data not shown).

Table 2. Carrier frequency of *CARD15* variants in patients with SpA, CD and controls (%).

Table 2.	SpA (*n*=104)				CD (*n*=156)				Controls (*n*=140)			
	R702W	G908R	1007fs	overall*	R702W	G908R	1007fs	overall*	R702W	G908R	1007fs	overall*
CARD15$^{-/+}$	12 (12)	4 (4)	4 (4)	**21 (20)**	39 (25)	11 (7)	27 (17)	**77 (49)**	18 (13)	1 (1)	6 (4)	**24 (17)**
CARD15$^{-/-}$	0 (0)	0 (0)	1 (1)		4 (3)	3 (2)	0 (0)		0 (0)	0 (0)	0 (0)	

Number of patients carrying R702W, G908R or 1007fs variants.
CARD15-/+: heterozygous; *CARD15*-/-: homozygous
Overall = total number of patients in the group carrying at least 1 variant
* The sum of all allelic *CARD15* variants is greater than the overall number of patients at least carrying one variant, since some patients carry 2 different SNP's, thus displaying a compound heterozygous status.

Table 3. Prevalences of *CARD15* polymorphisms according to the presence of mainly axial or peripheral involvement in the a) total SpA group (n=104), b) AS group (n=75) and uSpA group (n=29).

a) Total SpA group

	CARD15		
	wildtype	variant	total
axial	22	0	22
peripheral	61	21	82
total	83	21	104

p=0.006

b) AS group

	CARD15		
	wildtype	variant	total
axial	18	0	18
peripheral	41	16	57
total	59	16	75

p=0.006

c) uSpA group

	CARD15		
	wildtype	variant	total
axial	4	0	4
peripheral	20	5	25
total	24	5	29

p=1.0

In the CD population, a carrier frequency of 49% (77 of 156 patients) was observed (Table 1). Forty-three CD patients carried at least one R702W polymorphism, 14 patients carried at least one G908R polymorphism and 27 patients carried at least one 1007fs polymorphism. Fourteen patients carried two polymorphisms of which 7 patients were homozygous and 7 patients compound heterozygous (Table 2).
In the control group, 24 individuals (17%) carried *CARD15* polymorphisms (Table 1). All except one (compound heterozygous for the R702W and 1007fs variant) were single heterozygous (Table 2).
The prevalence of polymorphisms in the SpA cohort (20%) was not different from that observed in the control group (17%) (*P*=0.5, OR 1.22, 95% CI 0.64–2.34) and significantly lower compared to the prevalence found in our CD population (49%) (*P*<0.001, OR 3.85, 95% CI 2.17-6.83).

Association between CARD15 polymorphisms and intestinal inflammation in SpA patients.
The carrier frequency in the subpopulation of SpA patients with chronic gut inflammation was 38% (15 of 40 patients) which was significantly higher compared to the control population (*P*=0.006, OR 2.9, 95% CI 1.33-6.30) and the other SpA populations (*P*=0.001, OR 5.80, 95% CI 2.02-16.68) and not statistically different from that observed in our CD population (49%, *P*= 0.2, OR 1.62, 95% CI 0.80-3.31) (Table 1).
Of all SpA patients carrying *CARD15* polymorphisms, 71% (15 out of 21 patients) had chronic gut inflammation, 0% acute inflammation and 29% presented with normal histology (Table 1). The only SpA patient carrying 2 *CARD15* variants also had chronic gut inflammation. In contrast, only 25 out of 83 patients with a wild type genotype (30%) had chronic gut inflammation, 29% acute inflammation and 41% normal histology. Consequently, the presence of *CARD15* polymorphisms was associated with a higher risk for development of chronic gut inflammation.
There are no statistically significant differences between the AS and the uSpA group concerning the prevalence of *CARD15* polymorphisms in patients with normal (3/29 in AS vs 3/11 in uSpA, *P*=0.3, OR 3.3, 95% CI 0.5-19.4), acute (0/13 in AS vs 0/11 in uSpA) or chronic (13/33 in AS vs 2/7 in uSpA, *P*=0.7, OR 1.6, 95% CI 0.3-9.7) gut inflammation.
In the subgroup of 54 patients who were clinically reassessed, 4 patients evolved from histological chronic gut inflammation towards clinically overt Crohn's disease. Two of these 4 patients carried *CARD15* polymorphisms. The other 22 patients with chronic gut inflammation in this group did not develop clinical CD.

Association between CARD15 polymorphisms and HLA-B27in SpA patients
There was no significant association between the presence of these 2 genetic markers. Six of 34 HLA-B27 negative patients carried *CARD15* polymorphisms versus 13 of 47 HLA-B27 positive patients (*P*=0.3, OR 1.8, 95% CI 0.6-5.3).

Multivariate analysis
In the subgroup of 53 SpA patients of whom both HLA-B27 and HLA-B62 status were known, logistic regression was performed (with the presence of chronic gut inflammation as dependent variable). This showed that the association between chronic gut inflammation and *CARD15* polymorphisms (*P*=0.01, OR 17.3, 95% CI 2.0-152.3) is independent of HLA-B27 (*P*=0.42, OR 1.7, 95% CI 0.5-6.0) and HLA-B62 (*P*=0.28, OR 2.5, 95% CI 0.5-13.0)

DISCUSSION

This study describes a novel and remarkably strong association between variants in a host defence gene located on chromosome 16, *CARD15*, and a chronic form of gut inflammation in patients with spondyloarthropathies. Interestingly, the prevalence of *CARD15* polymorphisms in this subgroup of SpA patients was not significantly different from that observed in patients suffering from Crohn's disease.

Three single nucleotide polymorphisms have been associated with CD [21-23]. One variant (1007fs) encodes a truncated protein which results in altered activation of NF-κB in response to bacterial stimuli [29-31]. The two other single nucleotide polymorphisms (R702W and G908R) result in an amino acid substitution.

More recently, several groups assessed the linkage of *CARD15* variants in CD to particular clinical phenotypes but the results of these retrospective studies are disparate. The presence of two mutations has been linked to younger age at onset and preferential involvement of small bowel [43]. Preference for ileal involvement was also reported by Cuthbert [42] and by Ahmad [44]. Prevalence for fibrostenosing disease was dominant in a study of Abreu *et al* [45]. In these studies, no association of *CARD15* variants with extra-intestinal involvement could be retained.

The present study demonstrates a new association between these three CD-associated variants in the leucine rich region of the *CARD15* gene and a distinct subpopulation of patients with spondyloarthropathies. Similarly to previous reports, the overall prevalence of mutations in SpA patients was not statistically different from the prevalence in our control population [33-37]. However, unlike the previous studies, we identified a distinct clinical subgroup, characterised by the presence of chronic inflammatory gut lesions, with a remarkably high prevalence (38%) of *CARD15* polymorphisms, being not significantly different from the prevalence in the CD population (48%) and significantly higher compared to the control population (17%) and the other SpA patients. Previous studies from our group showed that in particular these patients with chronic gut inflammation were at risk for the progression to Crohn's disease [11, 12].

Striking in the present study, none of the SpA patients with only axial disease carried *CARD15* polymorphisms. Carriers of these polymorphisms all had (a history of) peripheral disease. This is in concordance with previous studies, where more chronic gut inflammation could be found in AS patients with peripheral disease compared to strict axial AS patients [11].

One previous study investigated *CARD15* polymorphisms in AS patients with CD and ulcerative colitis (UC) [54]. It did not show a higher prevalence of *CARD15* variants in AS patients with CD compared to idiopathic AS, AS with UC or healthy controls. However, the low prevalence of *CARD15* variants in the CD population with AS was not compared to the prevalence in a general CD population and it unexpectedly revealed a possible association between the G908R *CARD15* variant and AS patients with ulcerative colitis.

Moreover, in a recent study we found an association between *CARD15* polymorphisms and the presence of radiological sacroiliitis in CD patients, unrelated to the HLA-B27 status of these subjects. These data already pointed at a role for the *CARD15* gene in the link between gut and joint inflammation [46].

Our findings confirm the previous reported clinical, therapeutical and immunological links between SpA and CD and provide also genetic proof for the association between both diseases. Since the chronic gut inflammation in the majority of SpA patients remains asymptomatic, this might suggest that *CARD15* polymorphisms could be linked with the development of (subclinical) chronic gut inflammation rather than with CD as such.

The underlying pathogenetic mechanisms that could explain the phenotypic expression of *CARD15* mutations in SpA need to be investigated. *CARD15* encodes a cytosolic protein that could play a role in SpA by interference with transport of antigens by macrophages from mucosal surfaces to the joints [55]. *CARD15* seems to function as an intracellular receptor for bacterial components, where the C-terminal Leucine Rich Repeat domain (LRR-domain) is crucial for responsiveness. Cellular response to bacterial products was altered in HEK293T cells transfected with expression plasmids containing any of the three SNPs [29, 31]. Moreover, expression of *CARD15* in myeloblastic and epithelial cells is enhanced by pro-inflammatory cytokines and bacterial components via NF-κB [30, 31, 56]. This response is likely to mediate cytokine production including TNF-alpha suggesting that upregulation of *CARD15* may be part of a positive regulatory loop and facilitate the response of the host to pathogens. A genetically determined disturbed handling of bacterial products in the intestinal tract, leading to an altered transport of antigens by macrophages to synovial tissue, is an interesting hypothesis that should be investigated in spondyloarthropathy. A further identification and characterisation of inflammatory cells involved in gut and joint inflammation may also lead to new therapeutic targets.

In conclusion, a distinct phenotype associated with the three main CD associated *CARD15* variants is reported in patients with SpA. Our data show that the presence of *CARD15* variants in SpA patients strongly predisposes to chronic intestinal inflammation, defining a population at risk for evolution to CD. However, the persistence of the subclinical character of the inflammation in a large part of patients may reflect that CD is a multigeneic disease or alternatively that the heterozygous carriage of *CARD15* polymorphisms predisposes only to a subclinical inflammation.

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Competing interest statement:
The authors declare that there are no competing interests.

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